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Extracellular histones are major mediators of death in sepsis

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Abstract

Hyper–inflammatory responses can lead to a variety of diseases including sepsis1. We now report that extracellular histones released in response to inflammatory challenge contribute to endothelial dysfunction, organ failure and death during sepsis. They can be targeted pharmacologically by antibody to histone or by activated protein C (APC). Antibody to histone reduced the mortality of mice in lipopolysaccharide (LPS), tumor necrosis factor (TNF) or cecal ligation and puncture models of sepsis. Extracellular histones are cytotoxic toward endothelium *in vitro* and are lethal in mice. *In vivo*, histone administration resulted in neutrophil margination, vacuolated endothelium, intra–alveolar hemorrhage and macro and microvascular thrombosis. Histone was detected in the circulation of baboons challenged with *E. coli* and the increase in histone levels accompanied the onset of renal dysfunction. APC cleaves histones and reduces their cytotoxicity. Co–infusion of APC with *E. coli* in baboons or histones in mice prevented lethality. Blockade of protein C activation exacerbated sublethal LPS challenge into lethality which was reversed by antibody to histone. We conclude that extracellular histones are potential molecular targets for therapeutics for sepsis and other inflammatory diseases.

Macrophage activation leads to production of several mediators including TNF and high mobility group box–1 protein (HMGB1) that contribute to the severity of sepsis1.

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Author contributions

Jun Xu designed and executed many of the experiments, Xiaomei Zhang participated in the experimental execution and contributed to experimental design, Rosana Pelayo was involved in the initial experiments to activate the macrophages, leading to the release of histones, Marc Monestier provided the antibodies to the histones and contributed useful comments, Concetta Ammollo and Fabrizio Semeraro performed the CLP experiments, Fletcher Taylor provided the archival baboon sepsis plasma and contributed useful comments, Naomi Esmon made useful comments and assisted in manuscript preparation, Florea Lupu provided constructive critiques of the studies, performed the calcium flux experiments and the histochemical analysis of the tissues, and Charles Esmon oversaw the overall execution of the projects.

Recombinant human APC is approved by the FDA for the treatment of severe sepsis probably due to its anti–inflammatory and cytoprotective functions rather than its anticoagulant activity 2–6. To explore other physiological mediators involved in the pathogenesis of sepsis we cultured LPS and interferon gamma activated mouse macrophage RAW264.7 cells either in the presence or absence of recombinant human APC under the hypothesis that APC might proteolytically degrade an important mediator. The cytotoxicity toward endothelium was then compared between the two conditioned media. The medium from LPS and interferon gamma activated macrophages was toxic to the human endothelial cell line, EA.hy926, as measured by propidium iodide (PI) staining. APC reduced this cytotoxicity (Supplementary Fig.1a). Comparing these media by SDS–PAGE, three new bands of 10 kDa, 13 kDa and 15 kDa appeared in the presence of APC (Supplementary Fig. 1b). Sequencing identified the 10 kDa band protein as the mouse histone H4 (H4) internal sequence methyl–Lys20–Ile34.The 13 kDa protein matches the mouse histone H3 (H3) internal sequence Lys27–Lys36. The N terminal sequence of the 15 kDa band protein could not be determined by direct Edman sequencing. Following in gel tryptic digestion, MS/MS identified three peptide sequences that match the mouse histone H2A protein sequences Ala21–Arg29, His82–Arg88 and Val100–Lys118. These data suggested that extracellular histones are cytotoxic toward endothelium and that APC is cytoprotective by cleaving them. The H3 identification was confirmed by Western blotting using antibody to H3 (Supplementary Fig.1c). The apparent increase in histone fragments present in the conditioned medium of activated macrophages cultured with APC might indicate that APC could not only cleave the soluble extracellular histones in the medium but also the histones associated with the activated cells or DNA.

To determine if histones are toxic to endothelium and whether APC can reduce the histone cytotoxicity, we treated EA.hy926 with a mixture of histones or five individual histones. We found that a mixture of histones was cytotoxic to these cells and this toxicity was mainly due to histones H3 and H4 (Fig. 1a). Inclusion of APC reduced this cytotoxicity (Fig.1b). Histones have similar or greater cytotoxicity toward primary human endothelial cells (HUVEC) and APC also reduces this cytotoxicity (Supplementary Fig. 2). When incubated with endothelium, H4 elicited calcium transients which were blocked by an antibody to H4 (Supplementary Fig. 3).

To test whether APC could cleave histones in a purified system, we incubated the purified H3 or H4 with APC. These histones were cleaved in a dose dependent fashion (Fig.1c). Liposomes containing phosphatidylethanolamine (PE) enhanced histone cleavage by APC (Fig.1d), similar to the effect of PE on APC inactivation of coagulation factor Va7. This lipid mixture is presumably a mimic of a cell surface membrane after injury or exposure to a potent agonist.

Histone cytotoxicity is concentration dependent *in vitro* (Fig.2a). 10 nM and 100 nM APC reduced the cytotoxicity at low histone concentration (25 μg ml^{-1}) but only 100 nM APC effectively reduced the cytotoxicity of histones at 50 μg ml^{-1} (Fig.2a). Pre–incubation of histones (50 μg ml^{-1}) and APC (100 nM) for 5 min reduced cytotoxicity (Fig.2b). This cytoprotective effect of APC against histones is mediated by cleavage of histones (Fig.2c) and not by APC mediated PAR1 signaling, a known alternative cytoprotective function of

APC8, since APC was inactivated by PPACK after pre–incubation with the histones to prevent the APC mediated activation of PAR1. Protein C is converted to APC by the thrombomodulin–thrombin complex on endothelium. Endothelial cells were not protected with either protein C or thrombin from histone cytotoxicity, but were protected when both protein C and thrombin were present. Fully activated protein C provided the best protection (Fig.2d).

To test whether extracellular histones may be involved in the pathogenesis of diseases and if APC can cleave these histones *in vivo*, we examined frozen archival plasma samples from a non–human primate model of sepsis in which baboons were challenged with a lethal dose of *E. coli* 9. Infusion of APC rescued these animals9. We measured extracellular histones in the plasma from animals challenged with the lethal dose of *E. coli* either in the absence or presence of infused APC. Intact H3 was detected by Western blot in the plasma of two baboons challenged which reached ~15 μg ml^{-1} eight hours post–challenge (Fig.2e). We were unable to measure other histones by this method because those antibodies were not adequately sensitive. The increase in H3 accompanied the onset of acute renal failure as indicated by a high serum creatinine level, 2.65 ± 0.05 mg dL^{-1} (normal range: 0.7 –1.4 mg dL^{-1}) eight hours post–challenge. Both intact and cleaved H3 were observed in the plasma of two animals challenged with a lethal dose of *E. coli* and administered APC, indicating that APC can cleave extracellular histones *in vivo* (Fig.2e). APC co–infusion protected renal function as indicated by the normal serum creatinine level, 1.15 ±0.15 mg dL^{-1} at eight hours post–challenge. We also found high levels of extracellular histones in frozen archival plasma samples from some septic patients consistent with the presence of nucleosomes10. In at least one patient treated with APC, significant cleavage of H3 was observed (Fig 2f). Thus, APC cleavage of extracellular histones in the circulation appears to be a new mechanism contributing to its beneficial effects in sepsis.

To test the toxic effect of histones *in vivo*, we injected 75 mg histones per kg intravenously into mice. All mice (n = 5) died within one hour after injection (Fig.3a). Co–injection of recombinant APC (5 mg per kg) rescued all of the mice (n = 5) challenged with the same lethal dose of histones (Fig.3a). The ratio of APC to H3 used to rescue the mice *in vivo* is similar to that used in endothelial cytoprotection experiments. Mice challenged with a sublethal dose of histones (50 mg per kg) exhibited many of the characteristics of septic animals. *In vivo*, lungs exhibited neutrophil margination and accumulation in the alveolar microvessels (Fig. 3b–d), vacuolated endothelial and pulmonary epithelial cells (Fig. 3e–f), intra–alveolar hemorrhage (Supplementary Fig. 4a, b), and platelet and fibrin–rich microthrombi (Supplementary Fig. 4c, d, f and g), intra–alveolar fibrin deposition (Supplementary Fig. 4e) as well as fibrin and collagen accumulation within the inter–alveolar septum (Supplementary Fig. 4f–g).

To test the pathological significance of extracellular histones in the progression of the septic response, we co–infused antibody to H4 with a high dose of LPS (Fig. 4a). The antibody protected the mice, indicating that H4 is a major mediator of injury in sepsis. To test whether inhibition of histone cytotoxicity by endogenous APC does indeed play a significant role in protection from death in a model of sepsis, we challenged mice with a low dose of LPS in the absence or presence of an antibody to protein C. This antibody, which

blocks protein C activation both *in vitro* and *in vivo*11, converted a non–lethal into a lethal LPS dose (Fig. 4b). This result is consistent with the recent finding that acute inflammation is exacerbated in mice genetically predisposed to a severe protein C deficiency12 and clinical observations that acquired severe protein C deficiency is associated with early death in septic patients13. Co–infusion of H4 antibody rescued the mice from the lethality caused by LPS and the blockade of protein C activation (Fig. 4b). In contrast, the antibody to histone H2B failed to rescue the mice. H3 was detected in plasma from mice challenged with a lethal dose of LPS and the levels of the H3 were reduced by treatment with H4 antibody (Fig. 4c), demonstrating a role of H4 in facilitating histone release. When a sublethal dose of LPS plus an antibody to protein C were injected into mice, the levels of H3 were higher than mice injected with LPS alone (Fig. 4d), demonstrating a role of APC in regulating extracellular histone levels *in vivo*.

Cecal ligation and puncture (CLP) is often considered a good animal model of sepsis. Antibody to H4 also reduced the mortality of mice in the CLP model (Fig. 4e). We found that delay of treatment and the use of antibiotics were necessary to achieve a therapeutic benefit with the antibody treatment, perhaps because of the functions of histones in innate immunity14–16.

TNF can also be used to elicit a hyper–inflammatory state mimicking many aspects of sepsis17. Antibody to H4 reduced mortality in mice subjected to TNF injection (Fig. 4f).

Extracellular histones, mainly H3 and H4, appear to be both biomarkers of disease progression and therapeutic targets in sepsis and other inflammatory diseases. An effective histone blocking agent, such as the antibody described here, might prove therapeutic without the bleeding complications that can result from APC therapy. If effective, this approach is especially attractive in post surgical patients or patients with sepsis following trauma who are currently excluded from APC therapeutic intervention.

Methods

Reagents

Human protein C, bovine thrombin and rat monoclonal antibody to mouse protein C (MPC1609) were produced in our laboratory according to standard procedures18. Human recombinant APC (Xigris™) was purchased from Eli Lilly. Calf thymus histones (Sigma), calf thymus histone H1, H2A, H2B, H3 and H4 (Roche), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE) (Avanti Polar Lipids), LPS from Salmonella typhimurium (Lot 084K4104, Sigma), mouse recombinant interferon gamma (Biosource), mouse recombinant TNF (R&D Systems), goat antibody to histone H3 (Santa Cruz) and PPACK (Calbiochem) were also purchased. PS/PC (20:80) and PE/PS/PC (40:20:40) liposomes were prepared by membrane extrusion7. Mouse antibody to histone H2B (LG2–2) and H4 (BWA–3) were generated from autoimmune mice as previously described19.

Animals

6–12 week male C57BL/6 mice (Jackson Laboratory) were used according to an animal protocol approved by the Institutional Animal Care and Use Committee of the Oklahoma Medical Research Foundation. Baboon experiments were performed as previously described9.

CLP model

The CLP procedure was performed as described20. Briefly, after 16 hours fasting, mice were anesthetized by intraperitoneal injection of Avertin (0.25 g per kg) and the anterior abdominal wall was shaved. After mid–line laparotomy, the cecum was exposed, ligated right below the ileo–cecal valve without causing intestinal obstruction and then punctured twice with an 18G needle. A small quantity of intestinal content was extruded to ensure hole patency. The cecum was then placed back in the peritoneal cavity and the abdominal wall was closed in 2 layers. Treatment with gentamicin (5 mg per kg) plus antibody to H4 or mouse isotype control antibody (20 mg per kg) was done by intraperitoneal injection 6 hours after the end of the CLP procedure in order to let the abdominal infection become established and to mimic more closely the clinical situation.

Cell culture

The mouse macrophage cell line RAW264.7 cells were cultured in RPMI 1640 medium supplemented with 10% FBS. Human endothelial cell line EA.hy926 cells were cultured in DMEM supplemented with 10% FBS and HAT (hypoxanthine, aminopterin, thymidine). HUVEC were cultured in M199 supplemented with 10% FBS, endothelial cell growth factor and heparin as described18.

Histone cytotoxicity assay

EA.hy926 cells were incubated with concentrated RAW 264.7 conditioned medium or various histones mixed with or without 100 nM protein C, APC or 10 nM thrombin in Opti–MEM medium at 37°C for the indicated time and then for 5 min at room temperature after 10μg ml^{-1} PI was added. Cells were washed and detached with 0.526 mM EDTA in PBS and subjected to flow cytometry for PI staining.

Histochemistry

Mice were injected intravenously with 50 mg per kg of histones and were sacrificed after 3 hrs. Lung was collected and fixed with 4% paraformaldehyde for standard paraffin and cryo embedding or 2% glutaraldehyde and 3% paraformaldehyde in cacodylate buffer, pH7.2 for electro microscopy. Ultrastructural analyses were performed as described21. Hematoxylin–eosin and Mallory's phosphotungstic acid–hematoxylin staining for fibrin were done according to standard procedures. Immunofluorescence staining was performed on 10μm frozen sections from the lung of control and histone–treated mice. Antibodies included goat polyclonal IgG to mouse P–selectin (Santa Cruz), rabbit polyclonal IgG to fibrinogen (Dako), and rabbit polyclonal IgG to human neutrophil elastase (Dako). Antibody to rabbit IgG–FITC and antibody to goat IgG–Cy3 (Jackson Immuno) were used as detection antibodies.

Statistical analysis

Survival studies were analyzed using the log–rank test in the program Prism (GraphPad). Differences were considered statistically significant at a *P*–value <0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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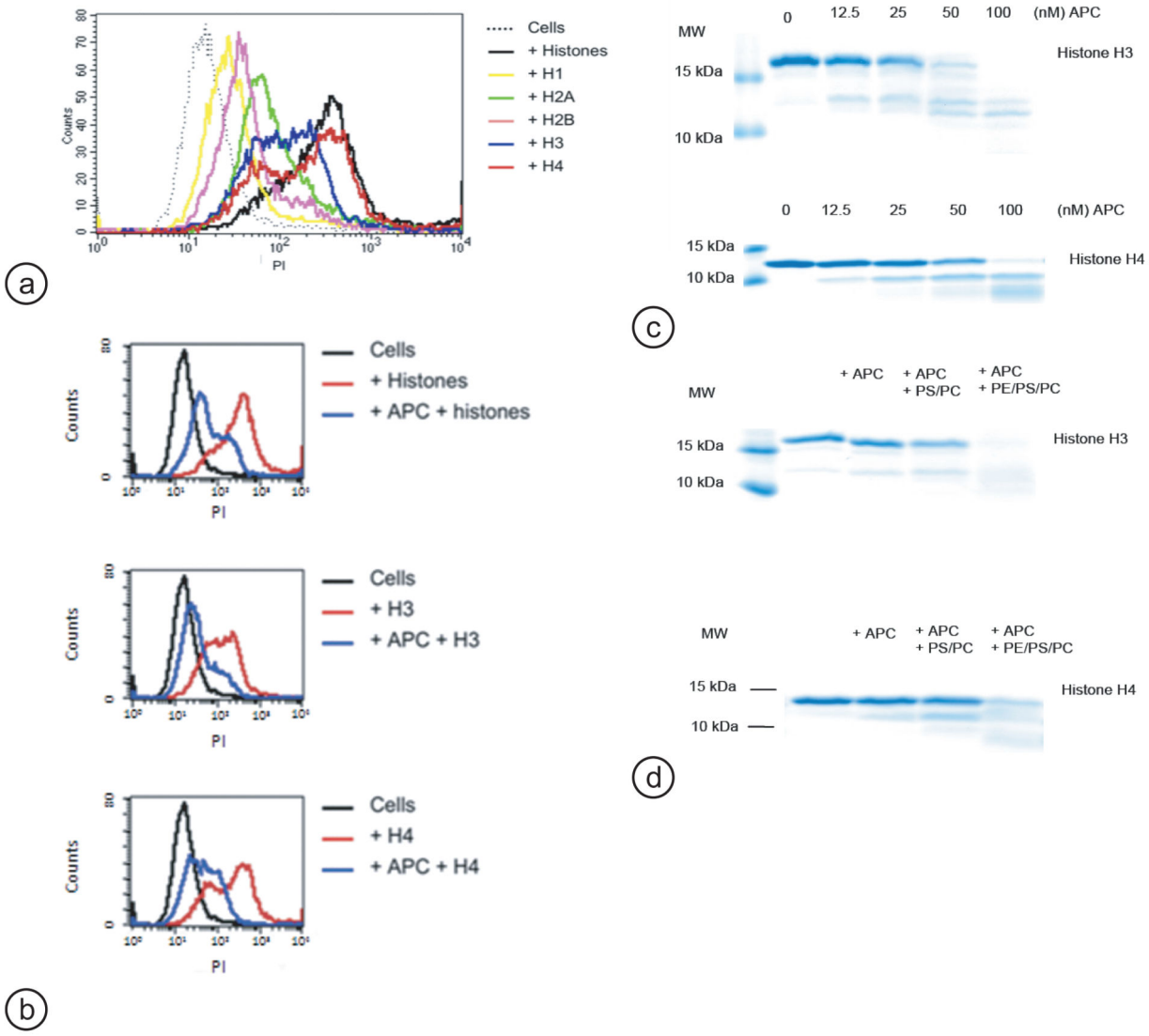

Fig.1.

Cytotoxicity of extracellular histones toward endothelium and APC cleavage of histones. (a) EA.hy926 cells were cultured with calf thymus histones (50μg ml^{-1}) or calf thymus histone H1, H2A, H2B, H3 or H4 (20μg ml^{-1}) for 1 hr at 37°C. Cell damage was measured by flow cytometry for PI staining. (b) APC (100 nM) was absent or present during the incubations with histones, H3 or H4 in the above assays. (c) SDS–PAGE analysis of purified calf thymus H3 (top panel) or H4 (bottom panel) (100 μg ml^{-1}) incubated with the indicated concentrations of human APC for 1 hr at 37°C. (d) SDS–PAGE analysis of purified calf thymus histone H3 (top panel) or H4 (bottom panel) (100 μg ml^{-1}) incubated with 10 nM human APC in the absence or presence of 0.5 mg ml^{-1} PS/PC or PE/PS/PC liposomes for 1 hr at 37°C.

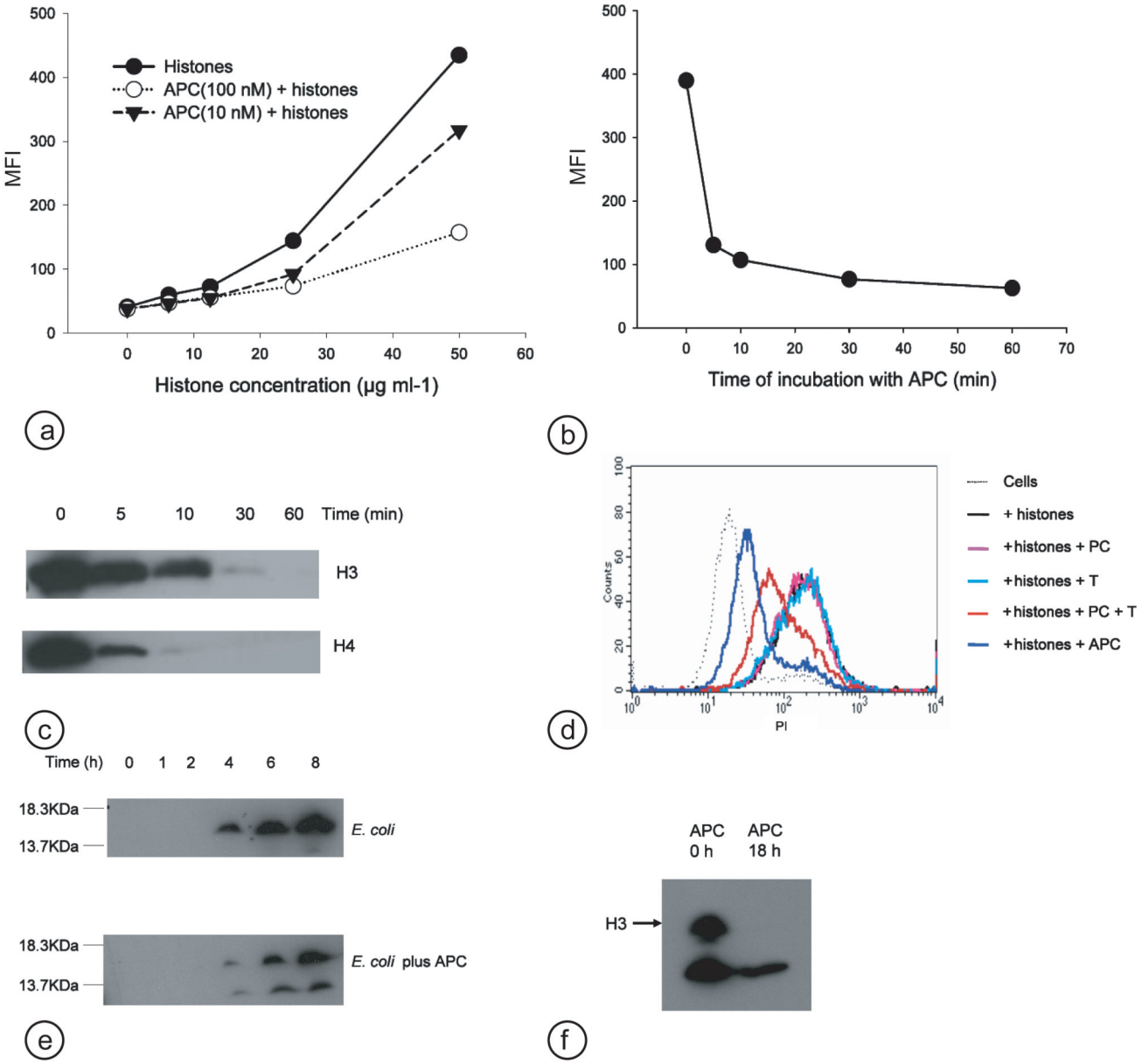

Fig.2.

APC cleaves histones both *in vitro* and *in vivo*. (a) EA.hy926 cells were cultured with calf thymus histones in the indicated concentration in the absence or presence of APC (10 or 100 nM) at 37°C for 1 hr. Cell damage was measured by flow cytometry for PI staining and expressed as mean fluorescence index (MFI). (b) Calf thymus histones (50 μg ml^{-1}) incubated with APC (100 nM) at 37°C for the indicated time and then mixed with PPACK (10 μM) to inactivate APC. The above medium was used to culture EA.hy926 cells for 1 hr for cytotoxicity assay or (c) subjected to SDS–PAGE and Western blotting for H3 or H4 at the times indicated. (d) EA.hy926 cells were cultured with calf thymus histones (50μg ml^{-1}) in the absence or presence of protein C (100 nM), thrombin (T) (10 nM) or APC (100 nM) at 37°C for 30 min. Cell damage was measured by flow cytometry for PI staining. (e) Western

blot analysis for H3 of baboon plasma samples at the times indicated after *E. coli* or *E. coli* plus APC challenge. (f) Western blot analysis for H3 of plasma samples taken at the times indicated from the start of APC treatment of a human septic patient.

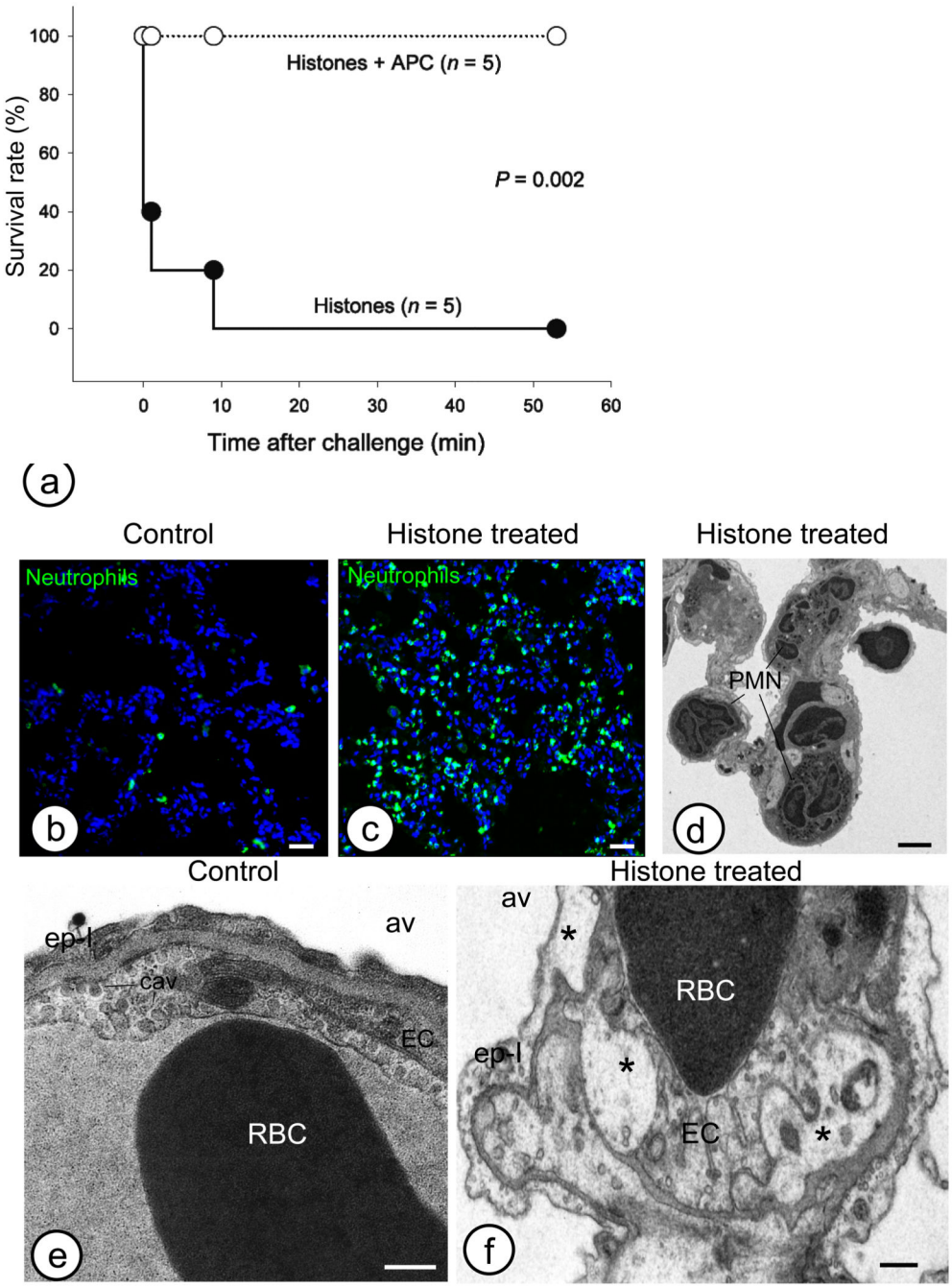

Fig.3.

Intravenous injection of histones elicits inflammatory and cell injury responses. (a) Survival rates of mice injected intravenously with calf thymus histones (75 mg per kg) with or without APC (5 mg per kg). (b–d) Pathological changes of mouse lung three hours after intravenous injection of histones (50 mg per kg). Immunofluorescence staining for neutrophil elastase detected massive neutrophil accumulation in the alveolar microvasculature (c: histone treated vs. b: control). Alveolar capillaries are almost fully obstructed by cells, as seen by electron microscopy (d: PMN). (e–f) Histones induce strong

alterations of the selective permeability of plasma membranes and subsequent intracellular edema, vacuolization (*) of the intracellular organelles (endoplasmic reticulum, Golgi, and mitochondria), both within endothelial (EC) and type I epithelial cells (ep–I). av, alveolae; cav, caveolae; RBC, red blood cells. Magnification bars: b and c: 50 μm; d, 10 μm; e and f, 500 nm.

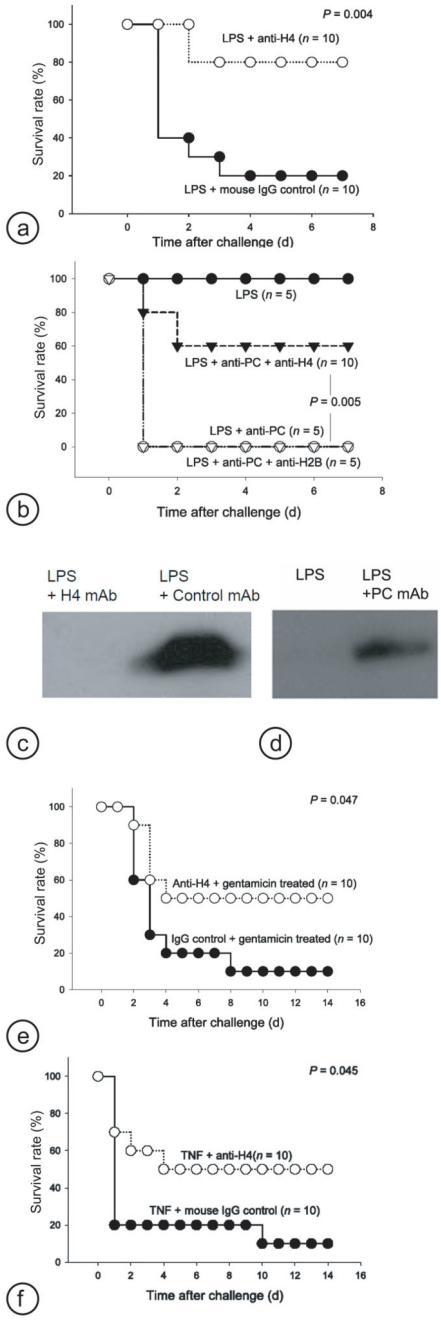

Fig.4.

Antibody to H4 protects mice from the lethality of LPS, CLP and TNF *in vivo*. (a) Mice were injected intravenously with a high dose of LPS (10 mg per kg) with antibody to H4 or mouse IgG control antibody (20 mg per kg). Survival rates of each group are indicated. (b) Mice were injected intravenously with a low dose of LPS (1 mg per kg) with or without antibody to protein C (2.5 mg per kg), and with antibody to H4 or H2B (20 mg per kg). Survival rates of each group are indicated. Western blot analysis for H3 of mouse plasma collected (c) 24 h after a high dose of LPS (10 mg per kg) plus antibody to H4 or mouse IgG

control antibody (20 mg per kg) or (d) 6 h after a low dose of LPS (1 mg per kg) with or without antibody to protein C (2.5 mg per kg). (e) Survival rates of mice subjected to CLP and then treated with antibody to H4 or mouse IgG control antibody (20 mg per kg) plus gentamicin (5 mg per kg) 6 hr post–CLP. (f) Survival rates of mice injected intravenously with TNF (0.75 mg per kg) plus antibody to H4 or mouse IgG control antibody (5 mg per kg).